

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A23J 1/00, C12P 19/24, 21/04, G01N 33/53		A1	(11) International Publication Number: WO 99/29182
			(43) International Publication Date: 17 June 1999 (17.06.99)
(21) International Application Number: PCT/US98/25734		(81) Designated States: BR, CA, JP, MX, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 4 December 1998 (04.12.98)			
(30) Priority Data: 08/985,548 5 December 1997 (05.12.97) US 08/986,234 5 December 1997 (05.12.97) US		Published <i>With international search report.</i>	
(71) Applicant: THE UNIVERSITY OF NEW MEXICO [US/US]; Patent Administration Office, Room 357, Hokona Hall, Albuquerque, NM 87131 (US).			
(72) Inventors: WALLEN, Erik, S.; 3901 Montgomery, N.E. #308, Albuquerque, NM 87109 (US). MOSELEY, Pope, L.; 9420 Eagle Rock Avenue, N.E., Albuquerque, NM 87122 (US).			
(74) Agent: JAGTIANI, Ajay, A.; Jagtiani & Associates, 10379-B Democracy Lane, Fairfax, VA 22030 (US).			
(54) Title: METHOD FOR PURIFYING HEAT SHOCK PEPTIDES COMPLEXES			
(57) Abstract			
<p>The present invention provides a method for synthesizing heat shock protein-peptide complexes comprising the steps of: adding a shock protein to a denatured protein matrix to bind the heat shock protein to the denatured protein matrix; and adding a complexing solution comprising a peptide to elute a heat shock protein-peptide complex. The present invention also provides a heat shock protein-peptide complex synthesized by the method of the invention. In addition the present invention provides an apparatus for synthesizing heat shock protein-peptide complexes comprising a heat shock protein complex bound to a denatured protein matrix. The present invention also provides a method for treating an allergic disease in which a heat shock protein-antigen complex is administered to a mammal in an amount sufficient to reduce the susceptibility of the mammal to a Th2 response for the allergic disease. The method of the present invention can be used either to prevent an individual from having an allergic reaction to an allergic disease or to reduce the effects of an allergic disease in an individual already suffering from the allergic disease.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

METHOD FOR PURIFYING HEAT SHOCK PEPTIDE COMPLEXES.

RELATED APPLICATIONS

5 The present application is a continuation-in-part of U.S. Patent Application Nos. 08/717,239 (U.S. Patent No. 5,747,332), and 08/934,139, the entire disclosure and contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

10

Field of the Invention

 The present invention relates generally to methods for synthesizing heat shock protein-peptide complexes. The present invention also relates generally to methods
15 for treating allergic diseases such as asthma and skin rashes using heat shock protein-based complexes.

Description of the Prior Art

20 Heat shock proteins (HSPs) are associated in cells with a broad spectrum of peptides, polypeptides, denatured proteins and antigens with which they form complexes. These complexes form as part of the cell's normal protein manufacturing process as described in Beckmann *et al.*, "Interaction of Hsp70 with newly synthesized proteins: implications for protein folding and assembly" in *Science* (1990), 248:850-4.
25 HSP-peptide complexes may also be formed as part of the transport of peptides to the MHC class I molecules on the cell's surface as described in Srivastava *et al.*, "Heat shock proteins transfer peptides during antigen processing" in *Immunogenetics* (1994), 39:93-98. When purified from autologous tumors, such HSP-peptide complexes have been useful in vaccines against cancers and infectious diseases as described in
30 Srivastava *et al.*, "Heat shock protein-peptide complexes in cancer immunotherapy" in

Current Opinion in Immunology (1994), 6:728-732; and Srivastava, "Peptide-Binding Heat Shock Proteins in the Endoplasmic Reticulum" in *Advances in Cancer Research* (1993), 62:153-177. Vaccination with antigenic peptides alone can elicit an immune response but peptides bound to HSPs appear to elicit a much more efficient response. In this regard, Srivastava has estimated in Srivastava, "Heat shock proteins in immune responses to cancer: the fourth paradigm" in *Experientia* (1994), 50:1054-1060, that 10 μ g of gp96 isolated from tumor cells may be complexed with approximately 10pg of peptide representing a tumor-associated mutation. While 10pg of peptide cannot be used to successfully vaccinate an animal against a subsequent tumor challenge, 10pg of peptide complexed with gp96 is enough to elicit protection.

Several types of cancer vaccines that are currently being investigated, including heat shock protein vaccines, have been described in Old, "Immunotherapy for cancer" in *Scientific American* (1996), 275:142. These potential vaccines have included: inactivated cancer cells and their extracts which can jump-start the immune system (particularly cancer cells engineered to secrete cytokines, such as IL-2 or GM-CSF, which similarly heighten anti-tumor immunity, and cells designed to express co-stimulatory molecules, such as B-1, which enhance the ability of T-cells to recognize tumor cells); tumor peptides, fragments of tumor proteins recognized by T-cells, which can be injected alone or with immune-boosting adjuvants; injected tumor proteins which are taken up by antigen-presenting cells and are broken down into a range of peptide fragments recognized by T-cells; dendritic cells, antigen-presenting cells which are isolated from the blood, exposed to tumor peptides or engineered to produce tumor proteins and then reinjected; gangliosides, which humans can produce antibodies against and which are found on the surface of tumor cells; viral and bacterial vectors, genes coding for tumor antigens which are incorporated into viral or bacterial genomes, that, when injected, draw immunity against themselves and encoded antigens; and DNA and RNA coding for tumor antigens which prompt normal cells to begin producing these antigens.

30

The heat shock protein vaccines consist of HSP-peptide complexes purified from tumor cells containing a mixture of peptides as described by Udono and Srivastava in "Heat shock protein 70-associated peptides elicit specific cancer immunity" in *Journal of Experimental Medicine* (1993), 178:1391-6. Most of the peptides complexed with HSPs and isolated from tumor cells are presumably portions of normal cellular proteins. Therefore, the HSP-peptide complexes need to be purified at fairly high levels in order to obtain HSPs complexed with peptide portions of mutated oncogenes. Because many of the purified peptides are portions of normal cellular proteins, vaccination with these purified complexes may incur the risk of auto-immune disorders.

10

Peptide vaccination experiments use peptide sequences of known tumor mutations such as described in Gjertsen *et al.*, "Vaccination with mutant ras peptides and induction of T-cell responsiveness in pancreatic carcinoma patients carrying the corresponding RAS mutation" in *Lancet* (1995), 346:1399-1400. Peptide vaccination experiments are also being tried with viral diseases such as described in Kelleher *et al.*, "Safety and immunogenicity of UB1 HIV-IMN octameric V3 peptide vaccine administered by subcutaneous injection" in *AIDS Research & Human Retroviruses* (1997), 13:29-32 are currently receiving much attention.

15

HSP-peptide complexes have been purified from autologous tumors and used for vaccination against the same tumor. It has been anticipated that material purified in this way can also be used for vaccination against human tumors in Srivastava and Udono, "Heat shock protein-peptide complexes in cancer immunotherapy" in *Current Opinion in Immunology* (1994), 6:728-32. The use of these purified complexes in treating patient tumors would require the isolation of tumor material from each individual patient. This is not practical or even possible for many types of tumors. Many types of cancer, however, have well-defined mutations in specific genes. The use of *de novo* HSP-peptide complexes allows the use of HSP-peptide vaccines without the need for removing a portion of the tumor. The *de novo* complexes also allow prophylactic

20

25

vaccination against a variety of diseases using peptides representing known oncogenic mutations or peptides representing portions of known viral proteins.

Bacterial hsp70-peptide complexes, created by incubating purified HSPs together with a synthetic peptide solution have also been used to stimulate peptide specific T-cell responses (See Roman *et al.*, "Synthetic peptides non-covalently bound to bacterial hsp70 elicit peptide specific T-cell responses *in vivo*," *Immunology*, (1996), 88:487-492, and Roman *et al.*, "Delayed-type hypersensitivity elicited by synthetic peptides complexed with *mycobacterial tuberculosis* hsp70," *Immunology*, (1997), 90:52-56).

In general, previous heat shock protein purifications have had two goals. The first goal has been to obtain pure HSPs without any contaminating peptides or proteins as described in Welch *et al.*, "Rapid purification of mammalian 70,000-dalton stress proteins: affinity of the proteins for nucleotides" in *Molecular and Cellular Biology* (1985), 5:1229-1237 and in Nandan *et al.*, "A rapid single-step purification method for immunogenic members of the hsp family: validation and application" in *Journal of Immunological Methods* (1994), 176:255-263. The second goal has been to purify the intracellular HSP-peptide complexes as described in Udonio *et al.*, "Heat shock protein 70-associated peptides elicit specific cancer immunity" in *Journal of Experimental Medicine* (1993), 178:1391-1396.

However, the fact that previously described methods only produce HSPs already complexed with intracellular peptides or stripped of their associated peptides is a serious deficiency. Also, there is no known simple way of synthesizing purified HSP-peptide complexes.

Numerous ways have been used in the past to treat allergic diseases such as desensitization therapy using allergy shots. For example, in the case of an individual suffering allergic rhinitis, the patient is given allergy shots containing antigenic peptides

or proteins which appear to shift the response from IgE to IgG production or to induce T-Cell mediated suppression (See Kuby, J., *Immunology*, (New York: W.H. Freeman, 1994) p. 432. However, the number of allergic diseases which can be treated using desensitization therapy is limited and it has been shown to have no value in treating many allergic diseases such as asthma.

The current mainstay of allergic disease therapy is the administration of topical (including inhaled) or system corticosteroids. These drugs work by globally blunting the immune response in an individual. Unfortunately, they have severe side effects. Because systemic corticosteroids blunt the entire immune response, they immunosuppress a patient increasing their vulnerability to infection. Corticosteroids also cause cataracts, bone demineralization, muscle wasting, weight gain, fluid and sodium retention, and mood disturbances such as insomnia, and steroid psychosis. Even inhaled steroids have been shown to retard growth in children and to cause a loss of calcium from the bones.

Other treatments for allergic diseases include the use of antihistamines and cromalyn like agents. However, these treatments only treat the symptoms caused by an allergic disease and do not address the immune response itself.

Therefore, there exists a need for an effective treatment of allergic diseases which can be used to treat numerous diseases and which does not have undesirable side effects.

SUMMARY OF THE INVENTION

The present invention provides a method of creating HSP-peptide combinations that will increase the effectiveness of peptide-based vaccines and allow more flexibility of use than the current HSP-based vaccines which require isolation of material from a

portion of the tumor itself. Although several authors have described systems in which HSP vaccines might be used, including: Blachere *et al.*, "Heat shock protein vaccines against cancer" in *Journal of Immunology* (1993), 14:352-56; Heike *et al.*, "Heat shock protein-peptide complexes for use in vaccines" in *Journal of Leukocyte Biology* (1996), 5 60:153-8; and Srivastava, "Heat shock proteins in immune response to cancer: the fourth paradigm" in *Experientia* (1994), 50:1054-60; Roman *et al.*, "Synthetic peptides non-covalently bound to bacterial hsp70 elicit peptide specific T-cell responses *in vivo*," *Immunology*, (1996), 88:487-492; Roman *et al.*, "Delayed-type hypersensitivity elicited by synthetic peptides complexed with *mycobacterial tuberculosis* hsp70, *Immunology*, 10 (1997), 90:52-56; none of these authors have speculated on the usefulness of purifying the proteins and synthesizing the complexes in a single step.

In one embodiment, the present invention provides a method for synthesizing heat shock protein complexes comprising the steps of: adding a heat shock protein to a 15 denatured protein matrix to bind the heat shock protein to the denatured protein matrix and adding a complexing solution comprising a peptide to elute a heat shock protein-peptide complex.

The present invention further provides a heat shock protein-peptide complex 20 made according to the method of the invention. Preferably, the heat shock protein-peptide complex is an ADP-heat shock protein-peptide complex.

The present invention further provides an apparatus for synthesizing ADP-heat shock protein-peptide complexes comprising an ADP-heat shock protein complex 25 bound to a denatured protein matrix.

It is an object of the present invention to provide a treatment for allergic diseases which channels the immunoresponse rather than blunting it to leave a patient with a functional immune system to fight infections.

30

It is another object of the present invention to provide a treatment for allergic diseases which does not have severe side effects.

5 It is yet another object of the present invention to provide a treatment for allergic diseases which treats the allergic immune response directly, rather than just the symptoms.

10 The present invention provides a method for treating an allergic disease in which a heat shock protein-antigen complex is administered to a mammal in an amount sufficient to reduce the susceptibility of the mammal to a Th2 response for the allergic disease. The method of the present invention can be used either to prevent an individual from having an allergic reaction to an allergic disease or to reduce the effects of an allergic disease in an individual already suffering from the allergic disease.

15 Other objects and features of the present invention will be apparent from the following detailed description of the preferred embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be described in conjunction with the accompanying drawings,
in which:

5

Figures 1A to 1E are a table showing peptides suitable for use in a preferred
embodiment of the invention.

10

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

For the purposes of the present invention the term "peptide" refers to all types of
15 peptides and conjugated peptides including: peptides, proteins, polypeptides, protein
sequences, amino acid sequences, denatured proteins, antigens, oncogenes and portions
of oncogenes.

For the purposes of the present invention the term "individual" refers to either an
20 individual person or animal from whom a cell lysate, heat shock protein, or peptide is
obtained or an individual patient who is treated according to the method of the present
invention.

For the purposes of the present invention the term "allergic disease" is used
25 broadly to cover reactions to various antigens which cause a Th1 response and includes
such diseases as asthma, allergic rhinitis, and sinusitis; allergic conditions of the lung
such as eosinophilic pneumonia; allergic diseases of the skin, such as skin rashes due to
reactions to poison ivy, poison oak, bee stings, jellyfish stings, etc.; and allergic diseases
of other organs such as the eye, kidney, intestine, liver, etc.

30

For the purposes of the present invention, the term "mammal" includes human beings.

For the purposes of the present invention the term "antigen" includes peptide
5 and nonpeptide substances capable of inducing an immune response.

10

Description

In a preferred embodiment the invention provides a method for synthesizing HSP-peptide complexes. In this method, HSP complexes are bound to a denatured
15 protein matrix or column. Then a complexing agent, such as a peptide, a polypeptide, a denatured protein and/or an antigen is added to the denatured protein column to bind with the HSP complexes, thereby removing these complexes from the column and forming an HSP-peptide complex which is eluted.

20 A preferred denatured protein matrix for the present invention can be formed by adding gelatin-agarose beads to a standard chromatography column.

One method for obtaining purified heat shock proteins for the present invention is to use a gelatin-agarose method such as that described in Nandan *et al.*, "A rapid
25 single-step purification method for immunogenic members of the hsp family; validation and application," in *Journal of Immunological Methods* (1994), 176:255-263. In this method of purification, cell lysates containing heat shock proteins are mixed with gelatin-agarose beads to bind the heat shock proteins to the beads. ATP is then added to the beads to release purified heat shock proteins.

30

Instead of synthesizing pure HSP-peptide complexes, it is often desirable to elute the HSP-peptide complex as an ADP-HSP-peptide complex. As described in U.S. Patent No. 5,747,332, such ADP-HSP-peptide complexes are stable and may be used to develop vaccines or immunotherapeutic tools for tumors and for infectious diseases.

5 These ADP-HSP-peptide complexes can be formed in a number of ways. Preferably such complexes are formed by adding the heat shock protein to the denatured protein matrix in the form of an ADP-heat shock protein complex so that the HSP-peptide complexes synthesized in the column will include complexed ADP. To form the ADP-heat shock protein complex, the purified heat shock protein is mixed with ADP. A
10 solution containing the ADP-heat shock protein complex is then added to an agarose-gelatin matrix to bind the heat shock protein to the denatured protein or gelatin. Because the denatured protein in the column binds to a different site on the heat shock protein than the ADP does, the ADP remains complexed to the heat shock protein, even when it is bound to the denatured protein in the column. A peptide complexing solution
15 containing a peptide complexing agent is then added to the column to release the ADP-HSP complex as an ADP-HSP-peptide complex by binding to the peptide binding site on the HSP.

In addition to the process described above, there are a number of other ways of
20 insuring that ADP is complexed to at least some of the HSP-peptide complexes that are eluted. For example, ADP can be added to an HSP-containing solution, such as a cell lysate, before, after or at the same time as the HSP-containing solution is added to the denatured protein matrix. Also, where the HSP is added to the matrix as part of a solution or cell lysate containing ATP, ADP-HSP-peptide complexes can be synthesized
25 by converting ATP to ADP in the cell lysate either before or after it is added to the matrix. One method for converting ATP to ADP in a cell lysate is to add glucose and hexokinase to the lysate containing ATP and let the lysate sit at room temperature for a period of time. Other suitable methods for converting ATP to ADP are described in Lehninger, *Biochemistry*, (New York: Worth Publishers, 1970), p. 299.

30

Some of the peptides suitable for use as peptide complexing agents in the present invention include those described in U.S. Patent Nos. 5,348,864 (vav mouse oncogene); 5,320,941 (protein sequence of the mas oncogene and polypeptides derived therefrom); 5,614,192 (peptides capable of binding to T-cell receptors); and 5,550,214 (peptide
5 capable of binding in the HLA-A2 binding cleft and capable of stimulating proliferation of cytotoxic T-lymphocytes); the entire disclosure and contents of which are hereby incorporated by reference. Other suitable peptides are listed in Table 1 of Figures 1A to 1E.

10 The peptide complexing agents are typically obtained from purified cell lysates. The cells from which the peptide complexing agents are obtained can be from the same or different individual as the heat shock proteins to which they are complexed. The heat shock protein and peptide complexing agents can even be obtained from different species and either the heat shock protein or peptide complexing agent can be formed
15 synthetically.

Although there are many heat shock proteins that may be used in the method of the present invention, heat shock proteins that have proven particularly useful include members of the hsp60 family, hsp70 family, hsp90 family and the hsp104-105 family.

20

Members of the hsp60 family include hsp60, hsp65, rubisco binding protein, and TCP-1 in eukaryotes; GroEl/GroES in prokaryotes; and Mif4 and TCP1alpha and beta in yeast.

25

Members of the hsp70 family include DnaK proteins from prokaryotes, Ssa, Ssb, and Ssc from yeast, hsp70, Grp75 and Grp78(Bip) from eukaryotes.

Members of the hsp90 family include hsp90, g96 and grp94.

30

Members of the hsp104-105 family include hsp105 and hsp110.

In another preferred embodiment, the present invention provides an apparatus for synthesizing heat shock protein-peptide complexes. Such an apparatus may be created by adding a desired heat shock protein or ADP-heat shock protein complex to a denatured protein matrix, as described above for the method of the present invention, to form a column having heat shock proteins bound therein. Such a column can be used to form a desired HSP-peptide complex or ADP-HSP-peptide complex by adding a peptide complexing agent to the column to elute the HSP-peptide complex or ADP-HSP-peptide complex, respectively, as described above for the method of the present invention.

In addition, the present invention provides a method for treating asthma and allergic diseases using heat shock proteins (HSPs) and HSP-antigen complexes. The method is particularly useful where the antigen is a peptide.

The treatment is based on the fact that there are two categories of response to an antigen. One is a cell mediated response (Th1) and the other is a T-helper cell activation of lymphocytes resulting in an allergic type antibody mediated response (Th2). The response to any particular antigen tends to be either a Th1 or a Th2 response since each response down regulates the other.

The response of the immune system (Th1 or Th2) to an antigen is based in part on how the antigen is presented to the immune system. Soluble antigen, taken up by antigen presenting cells (APCs) and displayed on the surface of APCs results in a Th2 response to that antigen. Cell mediated responses are more complex and require the recognition of intracellular antigens displayed on the surface of the target cell.

It has been speculated that an organism's own HSPs serve as a danger signals which can alert the cell to the presence of viral infections or transformed cells (See

Matzinger *et al.*, "Tolerance, danger, and the extended family, *Annual Review of Immunology*, (1994), v.12, pp. 991-1045). It is believed that these danger signals, especially when they come from within the same species (i.e. human HSPs in a human, mouse in a mouse, etc.), favor a cell mediated response to an antigen (See Srivastava *et al.*, "Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations," *Science*, (1997), 278, 117-120). Therefore, allergy causing antigens presented as complexes with HSPs should result in a cellular reaction to those antigens which favors a Th1 environment and down regulates any Th2 response to that antigen.

10 The heat shock protein-antigen complex of the present invention can be administered in any conventional way for treating an individual with a drug or antigen such as by injecting a solution containing the complex into the individual, ingestion of a solution containing the complex, having the individual swallow a pill containing the complex, etc. Where the allergic disease causes a skin reaction, the complex of the present invention can be administered topically by applying a complex containing
15 solution to the surface of an individual's skin. The complex can be also administered through inhaling a misted solution containing the complex. The complex can be administered either proactively to a healthy individual to prevent the individual from having an allergic reaction or reactively to reduce the effects of an existing allergic
20 reaction in an individual.

 One preferred method of synthesizing the heat shock protein-antigen complexes of the present invention involves using a denatured protein column, as described above for the synthesis of heat shock protein-peptide complexes. In this method, HSPs (either
25 bound or unbound to ADP) are bound to a denatured protein matrix or column such as a gelatin-agarose column. Then a complexing agent comprising an antigen is added to the denatured protein column to bind with the HSP complexes, thereby removing these complexes from the column and forming an HSP-antigen complex which is eluted.

One method for obtaining purified heat shock proteins for HSP-antigen complexes is to use a gelatin-agarose method such as that described in Nandan *et al.*, "A rapid single-step purification method for immunogenic members of the HSP family; validation and application," in *Journal of Immunological Methods* (1994), 176:255-263.

5 In this method of purification, cell lysates containing heat shock proteins are mixed with gelatin-agarose beads to bind the heat shock proteins to the beads. ATP is then added to the beads to release purified heat shock proteins. These purified HSPs can then be mixed with antigenic peptides in order to form complexes. In order to facilitate binding, ADP can also be added to the mixture.

10

Another method for releasing the complexes would be to add antigen complexing solution containing a antigen to the column to release the ADP-HSP complex as an ADP-HSP-antigen complex by binding to the antigen binding site on the HSP. When the antigen is a peptide, the antigen binds to the peptide binding site on the
15 HSP.

Another preferred method of synthesizing heat shock protein-antigen complexes useful in the treatment method of the present invention involves adding heat shock proteins to an ADP matrix column to bind them to the matrix, adding a solution of
20 peptides, polypeptides, denatured proteins or antigens to the column to bind them to the heat shock proteins as heat shock protein complexes and then adding a buffer containing ADP to the column to remove the ADP-heat shock protein-antigen complexes in an elution product.

25 Naturally occurring heat shock protein-antigen complexes can be purified for use in the treatment method of the present invention by adding a solution containing heat shock protein-antigen complexes to a column containing an ADP matrix to bind the heat shock proteins complexes to the ADP matrix and then adding a buffer containing ADP to the column remove the ADP-heat shock protein-antigen complexes in an elution
30 product

In situations where it is undesirable to include ADP in the heat shock protein-antigen complex, heat shock proteins can be combined by is to mix HSPs and antigens in a container and let them sit for about 1 hour at about 37°C (See Roman *et al.*,
5 "Delayed-type hypersensitivity elicited by synthetic peptides complexed with mycobacterial tuberculosis hsp 70, *Immunology*, (1997) 90:52-56).

Although preferred methods of forming the heat shock protein-antigen complex used in the treatment method have been described above, other methods may be used to
10 form the heat shock protein-antigen complex.

The antigens which are bound to the HSP carrier are typically obtained from purified cell lysates. The heat shock protein and antigen may be obtained from the same cell, from different cells in the same individual, from cells in different individuals and
15 even from cells in different species. Also, either the heat shock protein or antigen can be formed synthetically, and the antigen may be recombinant or come from cell preps of allergy causing organisms.

20 The invention will now be described by way of example. The following example is illustrative and are not meant to limit the scope of the invention which is set forth by the appended claims.

EXAMPLE 1

25

Heat shock proteins from cells or sub-cellular fractions are solubilized in lysis buffer (20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1% Triton X-100) containing a cocktail of protease inhibitors. This cocktail consists of 25 µg/ml of leupeptin, 25 µg/ml of E64 antipain and 0.5 mM (polymethylsulfonyl fluoride). The lysate is centrifuged at 10,000
30 x g for 20 min. at 4°C. to remove insoluble materials. The supernatant is incubated with

appropriate amounts of gelatin-agarose beads by mixing for 2 hours at 25°C. This incubation will result in a gelatin-HSP(ADP)-peptide complex. The beads are collected by centrifugation and washed three times with a buffer adjusted to 0.5M NaCl, and once with 5mM Hepes, pH 7.5. Bound heat shock proteins are released by incubating the
5 gelatin agarose beads with a solution containing a peptide or peptides representing the tumor or viral antigen. The heat shock protein-peptide complexes can then be used as a vaccine. Examples of suitable peptides that can be bound to heat shock proteins includes those found in Table 1 of Figures 1A to 1E.

10

EXAMPLE 2

250 µg of synthesized Fel dI peptides are combined with 250 µg of purified mouse HSP70 for a total of 500 µg of HSP-peptide complex. Five B6D2F₁ mice are injected subcutaneously with 50 µg of HSP-peptide complex 10 days and 5 days prior to
15 antigen challenge. Five control mice are also injected with saline on the same days. All ten mice are challenged with antigen by injecting 100 µg of peptide in 0.2 mls of complete Freund's adjuvant in four sites at the base of the tail and in both thighs.

20

EXAMPLE 3

100 cat sensitive patients are randomly divided into treatment or placebo groups. Treatment groups are given an subcutaneous injection of 7.5 µg of HSP70-peptide complex. The injections occur once a week for 4 weeks.

25

Although the present invention has been fully described in conjunction with the preferred embodiment thereof with reference to the accompanying drawings, it is to be understood that various changes and modifications may be apparent to those skilled in the art. Such changes and modifications are to be understood as included within the

scope of the present invention as defined by the appended claims, unless they depart therefrom.

WHAT IS CLAIMED

1. A method for synthesizing heat shock protein complexes comprising the steps of:

adding a heat shock protein to the denatured protein matrix to bind the heat shock protein to the matrix; and

adding a complexing solution comprising a peptide to elute a heat shock protein-peptide complex.
2. The method of claim 1, wherein said heat shock protein added to the matrix is complexed with ADP.
3. The method of claim 2, wherein said eluted heat shock protein-peptide complex comprises an ADP-heat shock protein peptide complex.
4. The method of claim 1, wherein said heat shock protein is added to the matrix as part of a solution containing said heat shock protein and said method further comprises the step of adding ADP to said heat shock protein-containing solution.
5. The method of claim 4, wherein ADP is added to said heat shock protein-containing solution prior to said heat shock protein-containing solution being added to the matrix.

6. The method of claim 4, wherein ADP is added to said heat shock protein-containing solution after said heat shock protein containing solution is added to the matrix.
7. The method of claim 4, wherein ADP is added to said heat shock protein-containing solution at the same time as said heat shock protein-containing solution is added to the matrix.
8. The method of claim 1, wherein said heat shock protein is added to the matrix as part of a heat shock protein-containing solution which includes ATP and the method further comprises the step of converting ATP in the heat shock protein-containing solution to ADP.
9. The method of claim 1, wherein said heat shock protein is added to the matrix as part of a cell lysate.
10. The method of claim 1 wherein the heat shock protein comprises one of the group consisting of: hsp60, hsp65, rubisco binding protein and TCP-1 from eukaryotes; and GroEL/GroES, Mif4, TCPalpha and TCPbeta from yeast.
11. The method of claim 1 wherein the heat shock protein comprises one of the group consisting of: hsp104, hsp105 and hsp110.

12. The method of claim 1 wherein the heat shock protein comprises one of the group consisting of: DnaK proteins from prokaryotes; Ssa, Ssb, and Ssc from yeast; and hsp70, Grp75 and Grp78(Bip) from eukaryotes.
13. The method of claim 1 wherein the heat shock protein comprises one of the group consisting of: hsp90, g96 and grp94.
14. The method of claim 1 wherein the peptide is from the same individual as the heat shock protein.
15. The method of claim 1 wherein the peptide and the heat shock protein are from different individuals.
16. The method of claim 1 wherein the peptide and the heat shock protein are from different species.
17. The method of claim 1 wherein the complexing solution comprises a cell lysate.
18. The heat shock protein-peptide complex made according to the method of claim 1.

19. The ADP-heat shock protein-peptide complex made according to the method of claim 3.
20. An apparatus for synthesizing heat shock protein-peptide complexes comprising a heat shock protein bound to a denatured protein matrix.
21. The apparatus of claim 20, wherein said heat shock protein bound to the denatured protein matrix comprises a heat shock protein complexed with ADP.
22. The apparatus of claim 20 wherein said denatured protein matrix comprises a denatured protein column.
23. The apparatus of claim 20 wherein the heat shock protein comprises one of the group consisting of: hsp60, hsp65, rubisco binding protein and TCP-1 from eukaryotes; and GroEL/GroES, Mif4, TCPalpha and TCPbeta from yeast.
24. The apparatus of claim 20 wherein the heat shock protein comprises one of the group consisting of: hsp104, hsp105 and hsp110.
25. The apparatus of claim 20 wherein the heat shock protein comprises one of the group consisting of: DnaK proteins from prokaryotes; Ssa, Ssb, and Ssc from yeast; and hsp70, Grp75 and Grp78(Bip) from eukaryotes.

26. The apparatus of claim 20 wherein the heat shock protein comprises one of the group consisting of: hsp90, g96 and grp94.
27. A method for treating an allergic disease comprising the step of administering a heat shock protein-antigen complex to a mammal in an amount sufficient to reduce the susceptibility of the mammal to a Th2 response for the allergic disease.
28. The method of claim 27, wherein said heat shock protein-antigen complex comprises a heat shock protein-antigenic peptide complex.
29. The method of claim 27, wherein said heat shock protein-antigen complex is administered by injecting said complex into the mammal.
30. The method of claim 27, wherein said heat shock protein-antigen complex is administered by inhaling a spray containing said complex.
31. The method of claim 27, wherein said heat shock protein-antigen complex is administered to the mammal by having the mammal ingest a solution containing said complex.

32. The method of claim 27, wherein said heat shock protein-antigen complex is administered to the mammal by having the mammal ingest a solid mixture containing said complex.
33. The method of claim 27, wherein said heat shock protein-antigen complex is topically administered by applying a solution containing the complex to the mammal's skin or to the mammal's eyes.
34. The method of claim 27, wherein said heat shock protein-antigen complex is administered to the mammal by having the mammal inhale a spray containing the complex.
35. The method of claim 27, wherein the mammal is a human being.
36. The method of claim 27, wherein said heat shock protein-antigen complex is administered to a mammal to prevent the mammal from having an allergic reaction to an allergic disease.
37. The method of claim 27, wherein said heat shock protein-antigen complex is administered to a mammal having an allergic disease to reduce the allergic reactions caused by the allergic disease.

38. The method of claim 27, wherein said heat shock protein-antigen complex comprises an ADP-heat shock protein-antigen complex.

1/5
Table 1

Protein	Amino Acid Sequence
Prostate Specific Antigen	Amino acids 146-154, 41-150 and 154-163
VP1 protein of foot and mouth disease	Amino acids 135-159
Leishmaniasis protein GP63	Amino acids 467-482
MAGE-1	EADPTGHSY
Fleine Immunodeficiency Virus Protein V3	RAISSWKQRNRWEWRPD RAISSWKQRN SWKQRNRWEW QRNRWEWRPD
Foot and mouth disease proteins	IMDRFVKINSLSPTHVIDL PFGHLTKLELPTDHH SIINNYMQQYQNSM
Mutant P53 tumor suppressor protein	TYSPALKNMFYQLAKTCPVQLKMFYQLAKTCPVQL KMFYQLAKTCPVQL FYQLATCPV FLQLAKTCPV
Human papillomavirus	Amino acids 49-57
Human immunodeficiency virus protein gp160	RIQRGPGRAFVTIGK RIHIGPGRAFYTTKN SITKGPGRVIYATGQ TLHMGPKRAFYATGN SIHIGPGRAFYTTGE
Human immunodeficiency virus peptide	RIHIGPGRAFYTTKN
Human immunodeficiency virus V3 peptides	SIRIGPGKVFTAKGG SISIGPGRAFFATTD RIHIGPGRAFYTTKN SLSIGPGRAFRTREI

Fig. 1A

2/5

Table 1 (continued)

	GIAIGPGRTLYAREK
	SITKGPGRVIYATGQ
	RVTLGPGRVWYTTGE
	SIHIGPGRIFYATGD
	SIYIGPGRFHTTGR
	RIQRGPGRFVTIGK
	GIHFGPGQALYTTGI
	STPIGLGQALYTTRG
	STPIGLGQALYTTRI
	RTPTGLGQSLYTTRS
Hepatitis C proteins	
NS5	MSYSWTGACVTPCAAF
viral core protein	GFADLMGYIPLVGAPL
	LMGYIPLVGA
mutated RAS	KLVVVGAXGVGKSALTI
peptide from chlamydia	TINKPKGYTGKE
trachomatis	
Human Immunodeficiency	
proteins	
Gag	HIVWASRFL
	ILGQLQPSL
	SLQTGSEEL
	ELRSLYNTV
	SLYNTVATL
	EIKDTKEAL
	TLNAWVKVV
	DLNTMLNTV
	DIAGTTSTL
	TLQEQIGWM

Fig. 1B

3/5

Table 1 (continued)

	EIYKRWILL
	IILGLNKIV
	FMMTACGV
	VLAEAMSQV
	PIDKELYPL
Pol	LLTQIGCTL
	PIETVPVKL
	ALVEICTEM
	GIRYQYNVL
	YIYQYMDDL
	KIEELRQHL
	ELHPDKWTV
	DIQKLVGKL
	KLKRGTKAL
	ELAENREIL
	ILKEPVHGV
	PLVKLWYL
	ELQAIYLAL
	YLALQDSGL
	ALQDSGLEV
	LIKKEKVYL
	HLEGKVILV
	ELKKIIGQV
	PLWKGPAKL
	LLWKGEGAV
	IIRDYGKQM
Vif	MIVWQVDRM

Fig. 1C

4/5

Table 1 (continued)

	RIRTWKSLV
	DLADQLIHL
	KIKPPLPSV
Env gp120	MICSATEKL
	IILSWDQSL
	KLTSLCVSL
	KLTSNTSV
	VITQACPKV
	IIVQLNTSV
	TLKQIASKL
	AIINMWQKV
Env gp41	FLGAAGSTM
	TLTVQARQL
	GIVQQQNNL
	AIEAQQHLL
	QLQARILAV
	RILAVERYL
	GIWGCSGKL
	QIWNHTTWM
	YIKLFIMIV
	FIMIVGGLV
	MIVGGLVGL
	GLRIVFAVL
	SIRLVNGSL
	RLVNGSLAL
	SLALIWDDL
	LIWDDLRS

Fig. 1D

5/5

Table 1 (continued)

	RLRDLLIV
	LLLIVTRIV
	LIVTRIVEL
	LLQYWSQEL
	ELKNSAVSL
	LLNATAIAV
Vpr	AIIRILQQL
	IIRILQQLL
Rev	YLGRSAEPV
	QILVESPTV
	ILVESPTVL
Vpu	QIAIVAALV
Nef	GMDDPEKEV

Fig. 1E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/25734

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A23J 1/00; C12P 19/24, 21/04; G01N 33/53

US CL : 435/7.1, 7.8, 70.1, 94; 530/412

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.8, 70.1, 94; 530/412

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Biosis, caplus, cmbase, medline, wpids, aps

search terms: heat shock protein peptide complexes, purification, methods, adp elution

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	PENG et al. Purification of immunogenic heat shock protein 70-peptide complexes by ADP-affinity chromatography. Journal of Immunological Methods. 1997, Vol. 204, pages 13-21, whole article.	1-26 ----- 27-38
Y	GAO et al. Effect of Constitutive 70kDa Heat Shock Protein Polymerization on Its Interaction with Protein Substrate. Journal of Biological Chemistry. 12 July 1996, Vol. 278, No. 28, pages 16792-16797, specially Introduction and Results.	1-26
Y	PALLEROS et al. hsp70-Protein Complexes. Journal of Biological Chemistry. 06 May 1994, Vol. 269, No. 18, pages 13107-13114, whole article.	1-26

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*g* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

26 FEBRUARY 1999

Date of mailing of the international search report

16 MAR 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized Officer

GEETHA P. BANSAL

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/25734

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SRIVASTAVA et al. Heat shock protein-peptide complexes in cancer immunotherapy. Current Opinions in Immunology. 1994, Vol. 6, pages 728-732, see whole article.	27-38
Y	SRIVASTAVA, P.K. Peptide-binding heat shock proteins in the endoplasmic reticulum: Role in immune response to Cancer and in antigen Presentation. Advances in Cancer Research. 1993, Vol. 62, pages 153-177, whole article.	27-38
Y	SRIVASTAVA et al. Heat shock proteins transfer peptides during antigen processing and CTL priming. Immunogenetics. 1994, Vol. 39, pages 93-98, whole article.	27-38